Genetic Markers Associated with Green and Albino Plant Regeneration from Embryogenic Barley Callus

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ABSTRACT

Genetic control of plant regeneration from cultured plant tissues has been documented for a number of species. The characterization and manipulation of loci that influence morphogenic responses may be useful for the development of highly regenerable germplasm or for physiological investigations. Quantitative trait loci (QTLs) for morphogenesis from barley cell cultures were identified on the basis of associations of mapped markers with the regeneration responses of 77 doubled haploid (DH) lines derived from the cross Steptoe/Morex. Two models were developed, one describing green plant regeneration, and one describing albino plant regeneration, measured as the numbers of green and albino plants regenerated per gram fresh weight of embryogenic callus. Approximately 62 and 12% of the observed variability for green and albino plant regeneration, respectively, was explained by the models. An independent data set was developed that consisted of the regeneration responses of 25 additional DH lines which were chosen randomly from the same segregating population. The models were tested for their ability to predict the responses of these independent DH lines. This study identified new QTLs for plant regeneration (one for green plants and at least one for albino plants), and confirmed previously reported associations of three QTLs with green plant regeneration.

Current techniques for the production of genetically transformed barley (*Hordeum vulgare* L.) plants are heavily dependent on the delivery of DNA into totipotent cells within cultured tissues. Such cells must be induced to differentiate into fertile plants following a mutagenic period of in vitro growth and selection. Transgenic cells of most barley genotypes frequently cannot be induced to differentiate into plants, or only albino plants can be recovered (for a recent review, see Lemaux et al., 1999). Ultimately, it will be desirable to introduce exogenous DNA without relying on tissues which are genetically and epigenetically unstable (for example, see Phillips et al., 1994; Bregitzer et al., 1998b); in the meantime, greater transformation efficiencies have been realized by improving existing tissue culture and transformation protocols.

One successful approach to barley transformation has been simply to utilize particular genotypes that are amenable to the requirements of transformation, such as Golden Promise (Wan and Lemaux, 1994) or Igri (Jähne et al., 1994). Another approach has been to modify the protocols used for culturing barley tissues; for instance, optimizing copper concentrations and autoclaving procedures have each been shown to increase the recovery of green plants by a factor of two (Dahleen, 1995; Bregitzer et al., 1998a). Increased copper levels combined with particular combinations of phytohormones, and the resultant improvement in regenerability, have been instrumental in the transformation of once-recalcitrant barley genotypes, and in increasing the efficiencies of transformation of amenable genotypes (Lemaux et al, 1999).

A genetic approach to improving plant regeneration would be selection for the accumulation of favorable alleles for regenerability into a single, presumably superior, genotype. Plant regeneration from cultured tissues has been shown to be under genetic control in a number of species, and genetic markers associated with plant regeneration have been identified in cereals such as barley (Komatsuda et al., 1995; Mano et al., 1996), wheat, (*Triticum aestivum* L.; Ben Amer et al., 1997), rice, (*Oryza sativa* L.; He et al., 1998), and maize (*Zea mays* L.; Armstrong et al., 1992). Several examples of genetic manipulations of regenerability exist. Regenerability characteristics have been transferred via hybridization and selection in maize (Armstrong et al. 1992) and in barley (Komatsuda et al., 1995). In the study by Komatsuda et al. (1995), a single locus was identified; however, a subsequent study identified three additional loci influencing plant regeneration from barley callus (Mano et al., 1996). Further work to identify and study the loci linked to these QTLs may lead to a greater understanding of the physiological processes involved in the growth and differentiation of somatic tissues. Syntenic relationships among cereal species (Van Deynze et al., 1995) suggest that the results of such studies will be broadly applicable to many species.

The objectives of this study were to identify QTLs affecting green and albino plant regeneration from barley callus. We identified at least two previously unreported QTLs for morphogenesis in cultured barley tissues, and provide confirmation of three of the four previously reported QTLs (Komatsuda et al., 1995; Mano et al., 1996). In addition, predictive models were developed and tested for use in marker-facilitated selection schemes.

MATERIALS AND METHODS

Explant Donor Growth Conditions

Seventy-seven DH lines and their parents were chosen randomly from the mapping population developed from the cross of Steptoe/Morex (Kleinhofs et al., 1993), and grown in growth chambers as previously described (Bregitzer et al., 1995). Three separate evaluations, separated by time, were conducted for each DH line. Because a single growth chamber could not accommodate all 77 DH lines, they were assigned at random to one of three growth chambers. Each of the three growth chambers included the Steptoe and Morex parents

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Published in Crop Sci. 41:173–179 (2001).

Abbreviations: QTLs, quantitative trait loci; DH, doubled haploid; gfw, gram fresh weight.

and three DH lines (12, 20, and 40) to permit monitoring of chamber-induced variation.

Callus Initiation and Plant Regeneration

Detailed descriptions can be found in Bregitzer et al. (1995). Briefly, callus was initiated by placing five immature zygotic embryos (1–3 mm in length) scutellum-down on a modified MS (Murashige and Skoog, 1962) medium supplemented with 3 mg L^{-1} (6.8 μM) 2,4-D. Developing calli were maintained in dim light (approximately 1 μmol m^{-2} s^{-1}) provided by shaded fluorescent lamps. Germinating zygotic meristems were removed from the developing calli 1 to 3 wk after the embryos were placed on the medium (initiation). Four weeks after initiation, embryogenic-appearing sectors from the four most vigorously growing calli were subcultured onto fresh medium and subsequently maintained by biweekly subcultures. Eight and 12 wk after culture initiation, sectors from each callus were transferred to regeneration media under brighter light (approximately 5 μmol m^{-2} s^{-1}) provided by unshaded fluorescent lamps. The fresh weight of callus transferred to regeneration medium was recorded.

Statistical Analyses and Model Development

The regeneration response of each DH line was determined by three separate evaluations. For each evaluation, the regeneration response was the arithmetic mean of the number of plants per g fresh weight (gfw) of callus that were recovered from each of 10 to 15 petri plates (Evaluations 1 and 2) or 15 to 20 petri plates (Evaluation 3). Data on plant regeneration were examined first for differences among lines via analyses of variance by PROC CATMOD (SAS Institute Inc., 1999) and then for associations with markers mapped by the North American Barley Genome Mapping Project (Kleinhofs et al., 1993). For this work, a 123-marker subset was used (kindly provided by P. Hayes, Oregon State Univ., USA) in two ways. First, phenotypic and genotypic data were analyzed via Mapmaker/QTL version 1.1 (Whitehead Inst., Cambridge, MA, USA) utilizing as a criterion of significance an LOD score of 2.0 or greater. Second, associations between the 123 polymorphic markers and the phenotypic data on regeneration were examined via linear regression on a per-evaluation basis. Markers were considered as candidates for model development if they had an LOD score of at least 2.0, and/or if they were shown to be significantly associated ($P < 0.02$) with regenerability in at least two of the three evaluations.

These data formed the dependent data set from which models describing regeneration characteristics could be developed. Potential models were developed for main effects and two-way interactions by the Mallow's C_p selection function of the PROC REG procedure within SAS (SAS Inst., Inc., 1999). Two "best" models were chosen (one for the prediction of green plant regeneration, and one for the prediction of albino plant regeneration).

Model Testing

These models were then tested for their predictive abilities using an independent data set consisting of the regeneration responses of an additional 25 DH lines, randomly chosen from the same Steptoe/Morex DH population. The Steptoe and Morex parents and the three DH lines 12, 20, and 40 were included in these analyses to facilitate comparisons of these data to that obtained from the 77 DH lines. The regeneration responses of the 25 DH lines were measured in two evaluations as described above, and compared with the predicted responses for these lines generated by the models chosen for green and albino plant regeneration.

RESULTS AND DISCUSSION

Transgressive Segregation for Plant Regeneration

The original Steptoe and Morex parents varied markedly for the ability of embryogenic callus to regenerate green plants, but were similar with respect to albino plant regeneration. Plant regeneration responses within some lines varied greatly among experiments; nevertheless, analyses of variance detected significant differences among DH progeny lines for green and albino plant regeneration ($P < 0.05$). Significant differences ($P < 0.05$) were detected also among growth chamber environments as revealed by the genotypes included to monitor environmental differences. Because of the random assignment of DH progeny lines among growth chambers in each experiment, these differences did not greatly effect phenotypic characterizations.

The regeneration responses of 8- and 12-wk-old callus were highly correlated, and only the responses of eight-week-old callus (Fig. 1) were considered in the following analyses. The numbers for green plant regeneration per gfw of callus ranged from 0.1 to 44.5, with a mean of 6.7 per gfw callus. Transgressive segregation occurred also for albino plant regeneration. Albino plant regeneration ranged from 0 to 18.5 per gfw callus, with a mean of 2.0 per gfw callus. Correlation between green and albino plant regeneration was $r = 0.52$ ($P = 0.001$); such a relationship may be expected based on the assumption that both green and albino plant regeneration have common physiological origins.

Identification of Loci Associated with Plant Regeneration

Identification of putative QTLs was based on the output from maximum likelihood (Mapmaker/QTL) and regression analyses. The results of both analyses were similar; putative QTLs are shown in Table 1 and in Fig. 2 and 3. Seven QTLs were identified for green plant regeneration by Mapmaker/QTL; all but abc174 and abg705 were identified also based on regression analysis. An additional QTL, ksud17, was identified based only on regression analysis. There were two QTLs associated with albino plant regeneration, glb1 and abc171; abc171 was identified on the basis of regression analysis only.

Given the correlation noted in this study for green and albino plant regeneration, and the reasonable expectation of common underlying physiological processes, one might have expected to detect more QTLs in common for green and albino plant regeneration. However, most of the DH lines in this study regenerated none or very few albino plants. If in fact albino plant regeneration is controlled in part by the same QTLs that control green plant regeneration, additional experiments that can more accurately measure genetic variability for albino plant regeneration must be conducted.

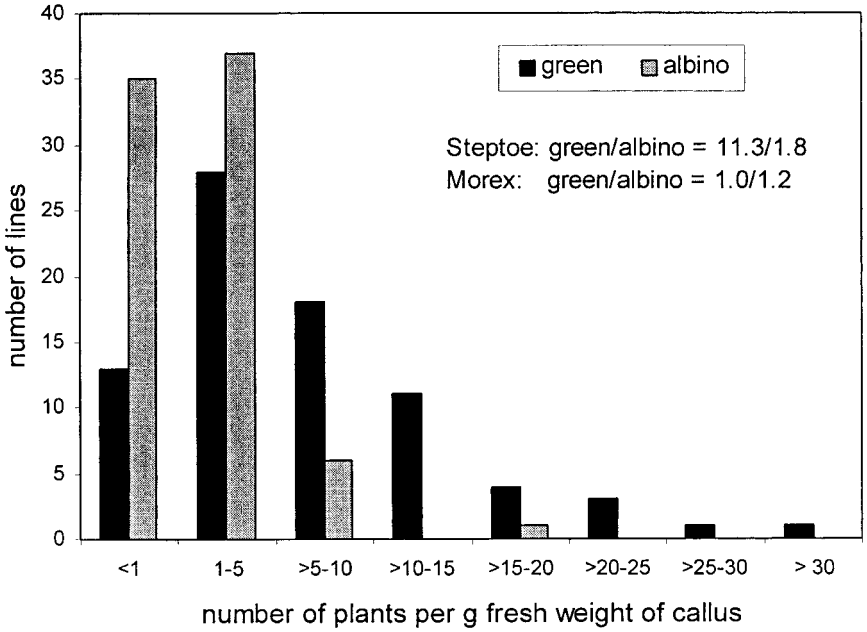

Fig. 1. Plant regeneration from embryogenic callus of doubled-haploid lines derived from Steptoe/Morex, including the responses of Steptoe and Morex.

We have developed a recombinant inbred population from a cross of 'Golden Promise' and 'Kanto Nakate Gold' and are examining the utility of this population for examining this issue. Both parents are characterized by very high rates of green plant regeneration, but differ markedly for albino plant regeneration.

The relatively low LOD scores obtained for several QTL peaks were unexpected. Previous studies in our laboratory had shown large experimental errors in the measurement of plant regeneration responses, and the decision was made to dedicate resources to multiple measurements on a subset of lines rather than single measurements of the larger 150 line population that was available. In retrospect this decision may have degraded the quality of our data, since single measurements of all 150 DH lines (Mano et al., 1996) produced generally higher LOD scores (for those QTLs detected both by this study and by Mano et al., 1996; see **Comparisons to Previously Published Studies** below). A theoretical treatment of this issue showed that increasing the number of individuals in the reference population, and therefore the number of potential recombinant genotypes, can be more important than increasing replication of phenotypic measurements (Knapp et al., 1990). An alternative explanation for low LOD scores may have been the observed variability between the multiple growth chambers used for plant growth, which would have increased the experimental errors of our models. Nevertheless, the data generated by our experiments appeared to be useful, and a careful reexamination of the data by regression analyses provided confirmation that the use of an LOD value of 2.0 was justified.

Model Development and Testing

Several putatively useful models were suggested on the basis of multiple regression analysis, and tested for their predictive abilities. The simplest model that appeared to be most predictive for green plant regeneration included four of the eight identified QTLs as main effects (psr129, abg019, dor4a, and abg705—see Table 2). Two additional QTLs (ksud17 and wg908) were included in two locus interactive effects, and resulted in a model that explained approximately 62% of the observed variability ($R^2 = 0.624$). The model that appeared most predictive of albino plant regeneration included both identified QTLs, and it predicted approximately 12% of the observed variability ($R^2 = 0.120$). The parameter estimates for significant main effects and interactions are shown in Table 2.

The responses of the 25 independently chosen DH lines were estimated based on the models presented in Table 2. For some DH lines breakpoints were present in the intervals indicated as QTLs by the models, which

Table 1. QTLs associated with green or albino plant regeneration from embryogenic callus of 77 DH lines derived from Steptoe/Morex, as identified by Mapmaker and/or regression analysis.

Trait	Marker interval†	Chromosome	LOD	Favorable allele‡
Green plants				
	abg461–*psr129*	1 (7H)	3.42	S
	abg019–abc162	2	5.25	S
	abc166–*abc174*¶,	3	2.55	M
	dor4a–abg471	3	2.48	S
	abg500b–abg472¶,	4	2.13	S
	ksud17§,	6	–	S
	abg705–abc483	7 (5H)	2.23	M
	wg908–abg495a	7 (5H)	2.28	M
Albino plants				
	glb1–abg494	5 (1H)	2.41	S
	abc171§,	3	–	S

† Italic typeface indicates best marker as identified by regression analysis.
‡ Letter indicates parental allele contributing the higher trait value; S = Steptoe, M = Morex.
§, ksud17 and abc171 identified by regression analysis only.
¶, Not included as a significant effect in the predictive model.

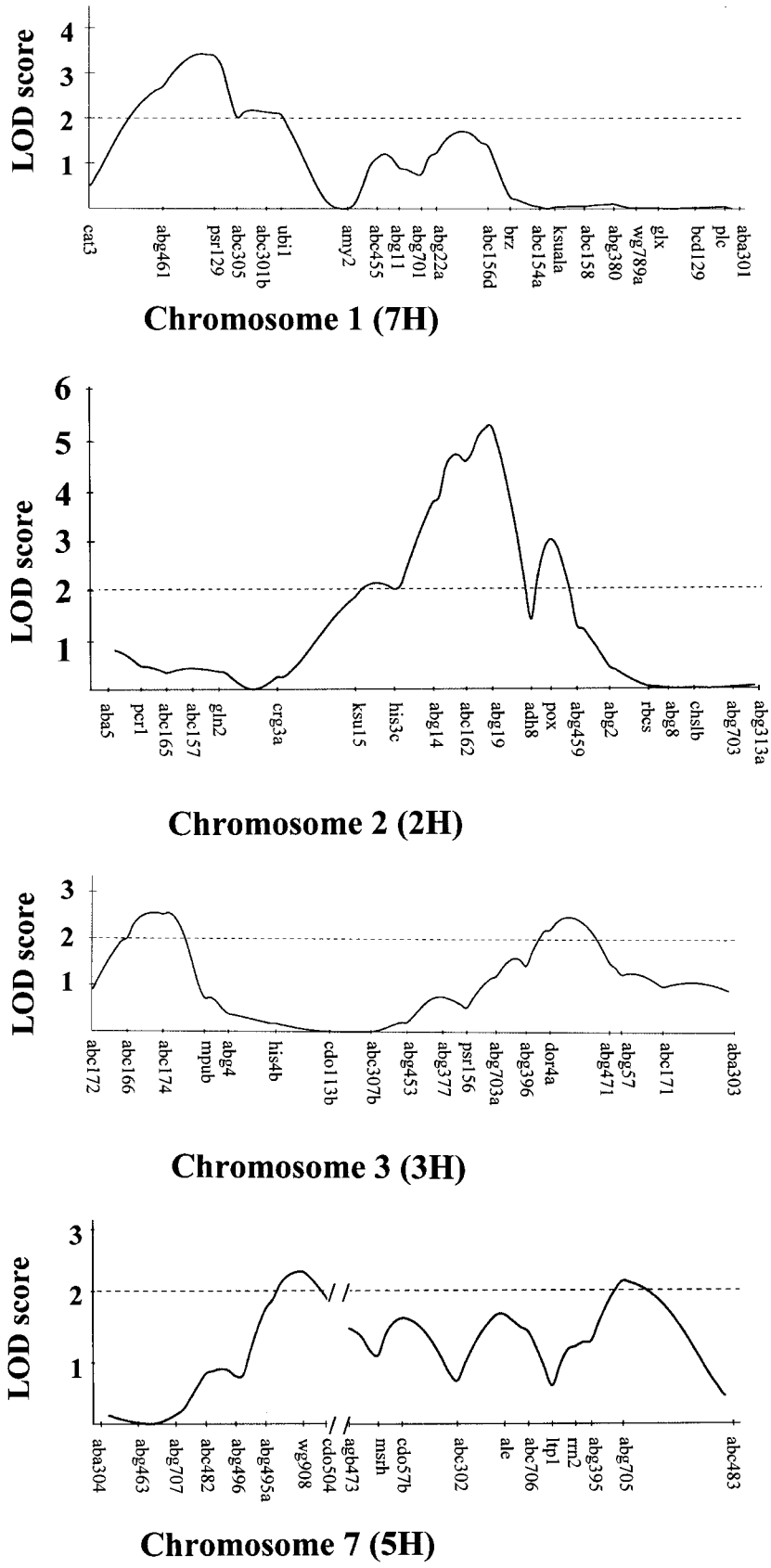

Fig. 2. LOD curves from QTL analysis green plant regeneration. For all chromosomes the distal end of the short arm is shown on the right. Note that for chromosome 7 (5H) Mapmaker/QTL 1.1 recognizes two linkage groups; cdo504 and abg473 are approximately 40 cM apart.

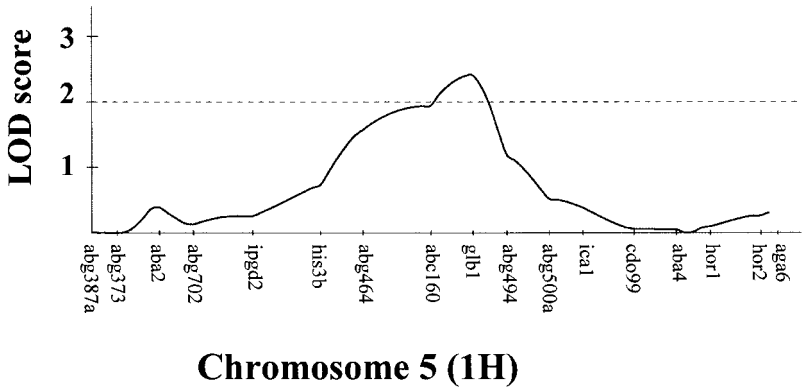

Fig. 3. LOD curves from QTL analysis albino plant regeneration; the distal end of the short arm is shown on the right.

prevented accurately determining the parental genotype at that QTL. Only those DH lines for which parental genotypes could clearly be assigned at each critical QTL were considered in assessing the predictive powers of the models. The predicted responses for green plant regeneration (n = 21 DH lines) and for albino plant regeneration (n = 22 DH lines) then were used to classify these DH lines into groups of high, intermediate, or low responders (with approximately equal numbers of lines in each group). To assess the potential for divergent marker-based selection, the observed mean responses of the predicted-high and predicted-low groups were determined, and compared with the observed mean responses of all the DH lines (21 or 22, for albino and green plant regeneration, respectively).

The range of regeneration responses for these DH lines were similar to those of the 77 DH lines used to generate the models, except that no lines were identified that had the very high levels of regenerability seen in several members of the 77-line data set. Green plant regeneration varied significantly ($P < 0.05$) and ranged from 0.3 to 19.7 plants per gfw callus, with a mean of 7.4 +/− 1.7. Albino plant regeneration varied significantly, and ranged from 0.1 to 2.5 plants per gfw callus, with a mean of 0.8 +/− 0.2.

For green plant regeneration, the predicted and observed means of the predicted-high group (n = 8) were 11.9 and 9.2, respectively. For the predicted-low group (n = 7), predicted and observed means were 1.2 and 4.6, respectively. For both the predicted-high and predicted-low groups, the observed means fell outside of the 5% confidence interval (CI) for the overall mean of the 21 DH lines (CI = 5.9 to 9.1).

For albino plant regeneration, the relatively simpler model generated only four predicted values. The highest (4.2, n = 5) and lowest (0.8, n = 6) predicted response groups were chosen for comparisons to observed responses. The observed means of the high and low predicted response groups were 1.2 and 0.9, respectively. The observed mean albino plant regeneration per gfw callus for all 22 lines was 0.8 +/− 0.2 (5% CI = 0.6 to 1.0). Only the predicted-high group mean observed response fell outside of the confidence interval for the overall mean.

Comparisons to Previously Published Studies

Komatsuda et al. (1991, 1993, 1995) and Mano et al. (1996) identified QTLs for green plant regeneration from barley callus. Mano et al. studied the same population (Steptoe/Morex-derived DH lines) as was studied for this report, whereas Komatsuda's group studied Japanese cultivars that are only distantly related to the U.S.-developed Steptoe and Morex cultivars. Comparisons of QTLs identified by Mano et al., Komatsuda et al., and in this study, were facilitated by reference to the consensus maps developed by Langridge et al. (1995) and by Qi et al. (1996), by the map produced by the North American Genome Mapping Project (Kleinhofs et al., 1993), and by data available on the Graingenes website at http://wheat.pw.usda.gov (verified September 7, 2000).

The QTLs for green plant regeneration linked to abg461-psr129 (7H) and abg705-abc483 (5H), and the QTLs for albino plant regeneration linked to glb1-abc494 (1H) and abc171 (3H), have not been previously identified. Although Mano et al. (1996) did not report the QTL linked to abg705-abc483, their reported LOD scores neared 2.0 in this region (see Fig. 5, Mano et al., 1996). It is interesting that the QTL linked to abg461-psr129 was not identified by Mano et al.; the relatively

Table 2. Model and parameter estimates for QTLs associated with plant regeneration from embyogenic callus based on data from 77 DH lines derived from Steptoe/Morex.

Trait	Model variable	Parameter estimate
Green plants		
	ps4129	−2.56
	abg019	−3.30
	dor4a	−1.44
	abg705	1.12
	psr129*abg019	1.40
	psr129*dor4a	0.92
	abg019*dor4a	0.87
	abg019*wg908	−0.77
	abg019*ksuad17	1.30
	dor4a*wg908	−0.89
	wg908*abg705	−1.40
	wg908*ksud17	−1.38
	abg705*ksud17	1.53
Albino plants		
	glb1	−0.88
	abc171	−0.81
	glb1*abc171	0.40

high (3.42) LOD score and importance of this allele in the predictive model suggests that this QTL has a major effect on green plant regeneration. Such a discrepancy between studies of the same population (Steptoe/Morex) may be attributed to differences in how plant regeneration was measured; Mano et al. (1996) measured plant regeneration as the percent of calli with plantlets, while this study measured plant regeneration as the numbers of plantlets per g fresh weight of callus. In our experience, these two measurements have not been tightly correlated (because of greater variability for total numbers of plantlets than for the percentage of calli with plantlets). Thus, we believe that measuring total numbers of plantlets provides a better characterization of regenerability.

The QTL for green plant regeneration linked to abg019-abc162 on chromosome 2H (this study) is probably analogous to the previously identified QTLs *Qsr1* and *Shd1*. Komatsuda and colleagues (Komatsuda et al., 1991, 1993, 1995) studied shoot regeneration in the progeny of a cross between a two-rowed and a six-rowed cultivar, and described the locus *Shd1* and its close linkage to the *V-v* locus that controls the fertility of lateral florets (2- versus 6-rowed heads); this region is within 10 cM of the QTL linked to the interval abg019-abc162 identified in this study. *Qsr1* (Mano et al., 1996) was placed between the markers abg316c and abc167b; abc162 (this study) is located within this interval. The detection of this QTL in the progeny derived from six-rowed parents (Steptoe and Morex; this study, and Mano et al., 1996) suggests that the QTL for regenerability is not the *V-v* locus.

This study identified QTLs linked to the interval dor4a-abg471 and to ksud17; these appear analogous to the markers identified by Mano et al. (1996) as *Qsr2* and *Qsr3* on chromosomes 3H and 6H, respectively. The QTL associated with albino plant regeneration detected in this study (abc171) is near the dor4a-abg471 interval identified in this study for green plant regeneration (approximately 17 cM; the confidence interval for dor4a-abg471 extends to abc171), and is coincident with *Qsr2*. Thus, these QTLs probably are not independent of each other and represent the same locus.

Mano et al. (1996) did not detect the QTLs identified in this study in the intervals abc166-abc174 on chromosome 3H nor abg500b-abg472 on chromosome 4H. These intervals were not included as significant effects in our predictive model and may be artifacts of experimental error.

There appears to be a QTL on chromosome 5H for which the location has not been adequately determined. In this study, a QTL was detected on chromosome 5H the interval wg908–abg495a, which at first glance appears unlinked to the interval cdo57b–msrh (*Qsr4*, Mano et al.), but these regions may not represent different QTLs. First, this comparison is complicated by the 40 cM gap between cdo504 and abg473. Second, the confidence interval (data not shown) for wg908-abg495a extended beyond cdo504 and nearly overlapped the confidence interval reported for *Qsr4*; the peak for *Qsr4* was broad (see Fig. 5, Mano et al., 1996). Finally, this study identified several other markers in this disputed region for which LOD scores neared significance. Additional data are needed to clarify the number and position(s) of QTLs for green plant regenerability on chromosome 5H.

CONCLUSIONS

This study has described new QTLs associated with the regeneration of green and albino plants from barley callus. The previously unidentified QTL (termed *Qsr5*, consistent with the terminology of Mano et al., 1996) linked to abg461-psr129 appears to have a major influence on the numbers of green plants regenerated. The QTL on chromosome 5H linked to abg705-abc483 likely represents a sixth (*Qsr6*) region which influences green plant regenerability. In combination with other QTLs identified in this study and by others (*Shd1*-Komatsuda et al.,1991, 1993, 1995; and *Qsr1*, *Qsr2*, and *Qsr3*-Mano et al., 1996), a model was proposed that predicted 62% of the variation for green plant regeneration. In practice, the model was able to roughly predict the green plant regeneration response (high versus low) of 21 additional barley genotypes derived from the Steptoe/Morex cross.

Only two QTLs were identified for albino plant regeneration. One (abc171) was linked to a major QTL (*Qsr2*) for green plant regeneration, suggesting an association with some basic aspect of morphogenesis that is not specific to the creation of albino plants; it probably does not represent an independent QTL. The QTL linked to the interval glb1-abg494 has not been previously identified; we have termed this QTL *Asr1* (albino shoot regeneration). The predictive model based on these QTLs explained only 12% of the observed variability for albino plant regeneration, suggesting that environmental factors played a relatively more major role in the regeneration of green plants than did genetic factors, and that this study was not able to accurately measure albino plant regeneration. Thus, the model describing albino plant regeneration may be of little practical utility for marker facilitated selection without further development.

These results demonstrate the feasibility of marker-facilitated selection for enhanced green plant regeneration. Cultivars such as Morex, which represents a commercially important germplasm pool that has poor regenerability characteristics, could be developed into genotypes that are elite for both regenerability and for commercial quality and agronomic characteristics. Additional efforts to more precisely locate and describe the nature of the genes these QTLs represent may lead to a greater understanding of the processes which regulate morphogenesis in barley and related members of the Gramineae family.

ACKNOWLEDGMENTS

The authors wish to express their thanks to Dr. Gary Richardson, for statistical consulting; to Drs. Diane Mather and Pat Hayes, for critical reviews of this manuscript; to Ms. Glenda Rutger, for technical assistance in the preparation of this manuscript; and to the North American Barley Genome Mapping Project, for financial support.

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